DEVELOPMENT OF PROBIOTIC TOFU PRODUCTS USING SELECTED SOYBEAN VARIETIES AND LACTOBACILLUS STRAINS

A

Synopsis

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1. Introduction

The high nutritional value and low cost of soybean products make them a suitable inexpensive way to overcome malnutrition problems among rural masses in India. Tofu, a fundamental part of Asian food culture, is a traditional oriental soybean food composed principally of protein and lipid. Currently, its consumption and popularity is not much more among poor people. Soy foods were also given a significant boost when FDA in 1999 approved a health claim linking soy foods with heart disease risk reduction (FDA, health claim petition, 2004). In fact, soy contains a number of biologically active compounds, including isoflavones, essential fatty acids, antihypertensive, antioxidative, opioid agonistic peptides and tocopherols (Adams et al., 2005; Andrade, Twaddle, Helferich, & Doerge, 2010; Martin et al., 2006). Tofu is the nutritive food made of soy has the potential health benefits. It is produced from water-extracted and salt- or acid precipitated soybean in the form of a curd, resembling a soft white cheese or a very firm yogurt. Tofu can also be further processed into various secondary tofu products, including deep-fried tofu, grilled tofu, frozen tofu, dried tofu, fermented tofu, and more. In most cases, these processed tofu products have different characteristics, such as taste, texture, end uses, and commercial identities with respect to the original plain tofu (Li & Hsieh 2004). Tofu is a perishable highly hydrated, gelatinous product having relatively high pH (5.8 to 6.2) and moisture content (80 to 88%) (Liu, 1997, pp. 1e 532). Its water content can be varied to produce an array of tofus with different characteristics.

Soybean, Glycine max (L.) Merrill belongs to family Fabaceae and subfamily Faboideae, globally known as *Golden Grain*. It is one of the world's most important oil producing crops having significant amount of protein, carbohydrates, fat etc. Soybean products have an excellent nutritional status based on their high protein content and all the essential amino acids to meet biological requirements (Naganagouda and Mulimani, 2006). Now cultivate in about 10 million hectare in India with a production of nearly 13.4 million tonnes. Soy vegetable oil is used in food industries; Soybean is the most important protein source for feed farm animals. In addition, it contains no cholesterol or lactose and only a small quantity of saturated fatty acid, and is suitable for those who are lactase deficient (Wang et al., 2002). Soybean seeds have a protein content of 35–40% on a dry weight basis, which makes them a relatively inexpensive source of protein for human consumption (Derbyshire, Wright, &

Boulter, 1976). Soybeans have been transformed into various forms of soy foods like soybean puffs, granules, oil, tofu etc. Among all these products tofu is the one most widely accepted throughout the world. However, it is not so common among poor people and rural masses in India.

Tofu is popular as **bean curd or soy paneer**, prepared by soy milk after coagulation and pressing the resulting curds into white blocks of varying softness; it can be *silken*, *soft*, *firm*, or *extra firm*. Beyond these broad categories, there are many varieties of tofu. It has a subtle flavor and spongy texture, so it can absorbs flavors very well. It is a traditional component of East Asian and Southeast Asian cuisines and has been consumed in China for over 2,000 years. Nutritionally, tofu is low in calories, while containing a relatively large amount of protein. It is high in iron, and can have a high calcium or magnesium content depending on the coagulants (e.g. calcium chloride, calcium sulfate, magnesium sulfate) used in manufacturing. Tofu is a well-known unfermented food product with nutritive and various health benefits (Rossi, Felis, Matrtinelli, Calcvecchia, & Torriani, 2016). Tofu has been distribute worldwide because of several potential health benefits to humans (Li,Qjao, & Lu, 2012; Yang et al., 2012). Western countries have started to take an increasing interest in tofu due to its good nutrition and health benefits to human.

Probiotics are live microorganisms that can be found in fermented foods and cultured milk, and are widely used for the preparation of infant food. They are well-known as “health friendly bacteria”, which exhibit potential health benefits and positively influencing the intestinal microbial balance’ (Crittenden et al., 2001). They boost up the micro flora of the gastrointestinal tract and helpful to enhance digestive system and immunity. Another benefit of the consumption of probiotics is their ability to reduce cholesterol absorption (Ooi and Liong, 2010). Probiotic bacteria are useful to mitigate lactose intolerance, treatment of diarrhea, anti-carcinogenic properties, reduction in blood cholesterol and improvement in immunity (Shah, 2007). Consumption of high concentration of probiotic bacteria per gram of product is required to health benefits (Ishibashi & Shimamura, 1993).

Probiotic bacteria are more and more often used in industrial production of fermented foods. Fermentation is used to conserve food in dairy, meat and fruit-vegetable industries. Probiotics are used in plant-derived products; include high nutritional and sensory values in these raw materials.

Probiotics enhance better assimilation of individual nutritional components; they synthesize some vitamins of B group and K. They increase absorption of minerals and vitamins.

The yield and quality of tofu are influenced by cultivar of soybeans (Shen, de Man, Buzzel, & de Man, 1991; Sun & Breene, 1001), varieties, soybean quality (growth and storage environment dependent), and processing conditions, methods (Saio, 1979 Shih, Hou, & Chang, 1997). Coagulation of the protein and oil (emulsion) suspended in the boiled soymilk is the most important step in the production of tofu making process but is complicated as the process depends on complex interactions. This process and the most difficult to master since it relies on the complex interrelationship of following variables: soybean chemistry; soymilk cooking temperature, volume, solid content and pH; coagulant type, amount, concentration and the method of adding and mixing; and coagulation temperature and time (Shurtle€ and Aoyagi, 1990; Cai and Chang, 1997). Tofu is thought to be a good medium for the growth of lactic acid bacteria due to a high protein content and pH close to neutral. During fermentation and growth of probiotic cultures a soy product becomes a product with functional properties (Jooyandeh, 2011). This process is accomplished with the aid of coagulants.

The present research work is dealing with the production of tofu from different varieties of soybean seeds. In this study a new way will be used to produce probiotic tofu by means of soymilk fermentation by specific strains of lactic acid bacteria (LAB), *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus paracasei* alone or in combination. In order to exceeding the shelf life and prevent undesired microbial and chemical spoilage, as well as improve the stability and the quality of the product some traditional techniques will be used.

In particular, the combination *L. casei* and *L. acidophilus* generated tofu having 20 days and able to prevent the growth of the spoilage strains. This fermented tofu was characterized by the production of antimicrobial molecules, such as acetic acid, limonene, 2-nonen-1-ol, 1-nonanol, 2(5H)-furanone, benzyl alcohol, phenylethyl alcohol and heptanoic acid. Depending on the *Lactobacillus* species used, the fermentation process generated different metabolites profiles and sensorial properties. Another promising properties conferred by the lactic acid bacteria fermentation was the inhibition of unsaturated fatty acids (UFAs) peroxidation or reduction of the aldehydes originated to their corresponding alcohols

However, soy-based foods also provide a range of health benefits to consumers due to their antihypertensive, hypocholesterolemic and antiartherogenic properties, and reduced allergenicity (Donkor et al., 2007). Various food products have been developed as carriers for probiotics, mainly of dairy origins because consumers commonly associate fermented dairy products with live cultures and perceive a benefit in the presence of these cultures (Sanders, 2000). Developing an efficient transformation system should be a prerequisite for both improvement of cultivar quality and investigations of the functional genes in soybean. Although over the past decade transgenic soybean plants have been obtained by the biolistic-mediated method (Finer and McMullen 1991; Hazel et al. 1998; Santarem and Finer 1999) and the Agrobacterium-mediated method (Hinchee et al. 1988; Zhang et al. 1999; Clemente et al. 2000; Yan et al., 2000; Olhoft and Somers 2001; Olhoft et al. 2001 Ko et al., 2003).

2. Objectives

1. Production of plain tofu from selected varieties of soybean seeds.
2. Production of probiotic tofu by using different strains of *Lactobacillus acidophilus, L. Casei, L. paracasei.*
3. Physical, chemical, microbiological and sensory analysis of plain and probiotic tofu.
4. Comparative study of plain, probiotic and locally available tofu.
5. To explore methods to extend the shelf life of tofu

3. Significance

Now a day, several soybean based products are very popular and easily available in market. They are inexpensive source of protein and have nutritional and therapeutic values as well. Present research work will be focused on such a tofu which has high nutritive and therapeutic potentials with extended shelf life. Actually tofu quality is based on quality of soybean seeds and microorganism used in the preparation of tofu. Here we will be used different strains of different species of *Lactobacillus* to enhance its value not only nutrition wise but therapeutically as well. As tofu quality is depend on quality of seeds so we will use different varieties of soybean seeds for tofu production.

This work includes extraction of soymilk and production of plain tofu from different varieties of soybean seeds. Different strains of *Lactobacillus acidophillous, L. Casei, L. paracasei* will be used to

prepare probiotic tofu which will be good to our digestive system and boost up the immunity. At present, shelf life of tofu is not much more; we will work to explore methods to extend the shelf life of probiotic tofu. The fermentation process increases the nutritional value of tofu, affect body immunomodulation and inhibit allergic reactions. High number of lactic acid bacteria make the product functional one and healthier in the way such as antibiotic includes diarrhea, lactose intolerance, irritable blower, immune system, digestion system, gut health. It will be high in protein content, cholesterol-free, low in saturated fat, so it has several potential health benefits for human consumption. Consumers can also consume probiotic tofu as a part of diet which may reduce cholesterol level and improvement in immunity. Probiotic tofu will be used for smoothies and desserts, soups, salads and make creamy sauces.

4. Review of literature

- **Dorota Zielińska et al., (2015)** worked on production methods for tofu with probiotic bacteria. Selection of a strain, production conditions, and a storage test are very important for developed product. It was concluded that the sensory quality of tofu with the addition of different probiotic cultures did not differ significantly and depending on used strains and their mixtures.
- **Ce Li, Xin Rui, Yuhui Zhang et al., (2017)** worked on fermented soy whey (Lactobacillus spp., streptococcus spp.) in production of tofu. Lactic acid bacteria were found as the major effective strains, and 7 lactic acid bacterial strains could produce enough acid to coagulate soymilk.
- **Sunantha Ketnawa1 et al., (2015)** worked on fish tofu was formulated by adding shrimp hydrolysate at levels of 0.05, 1.00, and 2.00 % (w/w) of ingredient formulation. The physicochemical, microbiological, and sensorial characteristics were evaluated for 14 days of storage at 4 °C.
- **Diana I. Serrazanetti et al., (2013)** studied the production of tofu by means of soymilk fermentation by specific lactic acid bacteria, Lactobacillus casei and Lactobacillus acidophilus alone or in combination, and subsequent precipitation has been developed, in

order to prevent undesired microbial and chemical spoilage, as well as improve the stability and the quality of the product.

- **Dong Li et al., (2006)** Thermal denaturation of soybean proteins is a prerequisite for tofu gel formation. Soymilk is conventionally heated with a one-stage steam heating. Two-stage ohmic heating was applied to soymilk in this study. Optimizing the two-stage heating condition for making filled-tofu and the effect of the two-stage heating on soft-tofu were investigated. Compared to the one-stage, two-stage heating increased the soft-tofu's apparent breaking strength by 12.2% and apparent Young's modulus by 16.2%, reduced the syneresis rate by 21.8%, and increased the yield and the solid recovery by 4.5% and 5.4%, respectively.
- **Memg Li, Fusheng Chen, Bap Yang et al., (2014)** worked on organic tofu using organic compatible coagulants of magnesium chloride and three polysaccharides including carrageenan, guar gum and gum Arabic were generated. For MgCl2 coagulated tofu, carrageenan significantly increased the hardness from 969.5 g to 1210.5 g whereas guar gum (0.6 g) decreased the hardness to 505.5g. Interestingly, gypsum and guar gum (0.6g) increased the yield of tofu significantly. These organic compatible coagulants didn't affect most of 75 and 115 protein subunits. Thus this mixture is promising as coagulant for making organic tofu.
- **Molamma P. Prabhakaran, et al., (2005)** reported the effect of different coagulants in their ability to retain isoflavones in prepared firm tofu. Harovinton beans were processed to obtain soymilk and a specific amount of this soymilk was coagulated using different types of coagulants for the preparation of tofu.
- **E.J. Noh a, S.Y. Park b (2004)** investigated the effects of freezing of soybeans on the coagulation of soymilk and quality of tofu. Soymilk, prepared from frozen soybeans, was found to coagulate faster in the presence of coagulant and produced a more uniform-structured gel than that from unfrozen soybeans. Tofu, prepared from frozen soybeans. There by inducing an increase in some textural parameters such as hardness, springiness, gumminess and chewiness as well as syneresis.

- **Xufeng Wang1,2 & Kaiyun Luo2 et al., (2018)** worked on contribution of soybean variety and coagulant type to the textural and rheological properties of soy protein isolate (SPI) tofu type emulsion gels was studied. SPIs from eight soybean varieties were subjected to amino acid and sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS-PAGE) analysis.
- **Young Soon Kim a, Yang Mun Choi b et al.,(2006**) studied the effects of oyster shell powder addition (0%, 0.05%, 0.1% and 0.2% of soy milk) on quality and shelf life of tofu (soybean curd) were investigated. Yield and moisture of tofu prepared with 0.1% of shell powder were higher than that coagulated with a single use of MgCl2. Shell powder addition (0.05% and 0.2% of soymilk) showed a high level in hardness and gumminess comparing with tofu prepared with a single use of MgCl2, No significant difference ($p > 0.05$) between 0.05% and 0.2% addition of shell powder.
- **Veronica A. Obatolu et al., (2007)** investigated soymilk tofu coagulated with four indigenous coagulants was compared in terms of chemical, textural, colour, and sensory attributes with calcium sulphate (CS) coagulated tofu. Coagulants used are Epsom salt, lemon juice, alum and top water of fermented maize. Protein and magnesium content significantly ($P< 0.05$) increased from 44.5 to 51.7 g/100 g and 252 to 324.6 mg/100 g, respectively, in soybean grain to soymilk. The hardness, chewiness and brittleness of textural properties of tofu were significantly ($P< 0.05$) affected by different sources of coagulation. This study has demonstrated that tofu quality is affected by the type of coagulant used in curding the soymilk.
- **Lei Ma, Bin Li1 et al., (2014)** studied soybean seed's chemical quality traits (including protein content, oil content, fatty acid composition, isoflavone content, and protein subunits), soymilk chemical character (soluble solid), and soymilk sensory attributes were evaluated among 70 genotypes to determine the correlation between seed chemical quality traits and soymilk sensory attributes. Six sensory parameters and a seven-point hedonic scale for each parameter were developed. Significant positive correlations were observed between overall acceptability and the other five evaluation parameters, suggesting that overall acceptability is an ideal parameter for evaluating soymilk flavor.

- **Woo-kyoung Shin, Wook Kim, et al., (2014)** worked on the fat content in SC-CO2 soy flour was reduced from 19.5% to 7.1%, which influenced some textural and sensory properties of soymilk and tofu. The viscosity of low-fat soymilk decreased from 50 to 40 cp. Low-fat tofu had a higher yield of 69.7%, compared to a yield of 60.8% for full-fat soymilk. The two types of tofu had similar flavor and texture intensity sensory scores, although low-fat tofu had a reduced score for roasted, nutty flavor than full-fat tofu. The pH of a low-fat tofu immersion solution during 30 days of storage was lower than for a full-fat tofu solution, particularly during the first 6 days.
- **Keat-Hui NG et al., (2008)** worked on the *Lactobacillus acidophilus, L. bulgaricus, L. casei, L. fermentum and Bifidobacterium bifidum* for their A-galactosidase activity over 24 h. L. bulgaricus and L. fermentum showed highest A-galactosidase specific activity and were selected to be incorporated into tofufa for a storage study of 9 days at 25 and 4 °C. The presence of probiotics in tofu showed an increase in the concentrations of organic acids which led to a decrease in pH levels.
- **S. Bin Md Yasir a,b et al., (2007)** worked on the enzyme transglutaminase was investigated for its cross-linking effect on the soy proteins of tofu. In vitro incubations confirmed that soy proteins are excellent substrates for transglutaminase, especially when denatured. The macroscopic effects resulting from the addition of transglutaminase were compared to changes at the microstructural and molecular level. Little, if any, cross-linking occurred within the tofu matrix in situ. This suggests that the change in functional properties afforded by addition of transglutaminase to tofu is due to a side reaction of the enzyme, for example hydrolysis of glutamine residues, rather than its cross-linking activity.
- **Rajani Mujoo et al., (2002)** studied the contribution of total soybean protein, the storage protein and their respective subunits to tofu yield and texture. Protein were analyzed by SDS-PAGE coupled with densitometry and received phase HPLC for seven soybean varieties, and correlated to tofu and texture.

- **Haizhen Moa,e, et al., (2013)** compared the content of bio accessible folate, vitamin B12, and isoflavones in tofu which strongly depending on bean variety. Folate and vitamin B12 contents were mainly influenced by microbial activity during fermentation.
- **Da-Young Lee a et al., (2016)** investigated the microbial contamination of commercial tofu from local supermarkets in Seoul, Korea. Growth modeling of Bacillus cereus isolated from spoiled tofu was used to determine the appropriate and temperature for safe storage of tofu. During the monitoring of 100 commercial tofu products, pathogenic bacteria (*Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Salmonella* spp*., and enterohemorrhagic E. coli*) were not detected. Aerobic bacteria were isolated from 32 of 100 tofu samples because of different sterilization and packaging methods.
- **Franca Rossia et al., (2016)** they reported on the microbiological characteristics of ready-to-eat fresh tofu produced by a small company in Northern Italy were investigated to unveil the abundance and composition of the microbiota and the identity and provenance of the specific spoiling microorganisms (SSO).
- **Ming-Ju Chen a at el.,(2003)** created response surface models through regression on experimental data and to apply the Sequential Quadratic Programming and Genetic Algorithms on the models to obtain optimal processing conditions for dairy tofu. The two-stage effort of obtaining a surface model using response surface methodology, and optimizing this model using GAs or SQP techniques was demonstrated to be an effective approach. Both SQP and GAs techniques were able to determine the optimal conditions for manufacturing the probiotic dairy tofu.
- **Woo-kyoung Shinet et al., (2014)** worked on the potential of supercritical CO2 (SC-CO2) extracted soy flour was evaluated as a raw material for functional foods. The fat content was reduced from 19.5% to 7.1%, which influenced some textural and sensory properties of soymilk and tofu. The viscosity of low-fat soymilk decreased from 50 to 40 cp. Low-fat tofu had a higher yield of 69.7%, compared to a yield of 60.8% for full-fat soymilk.

- **Chiung-Yuan Lee et al., (2010)** studied the effects of g-polyglutamates with different concentrations (0.1%, 0.15%, and 0.2%) and molecular weights addition on the rheological properties, microstructure, and syneresis of tofu. The addition of g-polyglutamate increased the gelation time, and decreased the storage modulus (G0) and the loss modulus (G00) of tofu. The molecular weight and concentration of g-polyglutamate effectively changed the rheological properties of tofu.
- **Shunhu Liu et al., (2007)** analyzed components and subunits based on distribution patterns of bands with varying molecular weights under SDS-PAGE. The number and molecular weight of the bands in SDS-PAGE varied among materials and showed a tendency of continuous distribution.
- **Debasree Ghosh et al., (2011)** worked on the cow milk curd was prepared using 2% of *Streptococcus thermophilus* and a mixed culture of *S. thermophilus* and *Lactobacillus plantarum* and incubating at 37 °C for 16 h. Improved functional and probiotic properties of curd. Structural changes in curd samples during fermentation were observed by Scanning Electron Microscope (SEM). Soy milk curd showed loosened structure. The degradation of proteins into peptides and amino acids were evaluated by SDS PAGE and amino acid analysis. Maximum production of amino acids i.e. cystine, histidine and asparagine were observed in both the cow and soymilk after fermentation.
- **Han-Sul Yang et al., (2006)** reported low fat sausages were prepared with added hydrated oatmeal or tofu as texture-modifying agents. The effects of the type and level of texture-modifying agents on the physical and sensory properties of low fat sausages were investigated. The water-holding capacity in sausage products increased by increasing the hydrated oatmeal level, but no significant differences was observed by the addition of tofu.
- **Jian-Yong Chuaa et al., (2019)** evaluated the current status of soy whey valorization that can be adapted from the dairy whey valorization. Various methods of soy whey utilization are discussed, which can be broadly classified into two categories: physical and microbiological/enzymatic. Physical methods include the recovery of the nutrients (e.g. isoflavone, protein and minerals) and the production of various functional ingredients (e.g.

emulsifiers), whereas microbiological/enzymatic methods include the production of functional ingredients using microorganisms and enzymes (e.g. prebiotic and citric acid), propagation of microorganisms (e.g. probiotics) as well as biofuel production.

- **Kyo-Yeon Le et al., (2018)** studied the yield and quality of low-fat tofu using defatted soy flour treated by supercritical-CO2 and hexane. Characteristics such as yield, textural attributes, color values, microstructure and storage stability of LFTs were measured and compared. SDS-PAGE showed that DF-SC had no protein denaturation, while DF-H had substantial denaturation. DF using SC-CO2 (DF–SC) had higher soluble solids in soy milk than in the control or that treated by hexane. DF–H. Tofu made from DF-SC had a softer texture than the other tofus, likely due to the relatively high moisture content.
- **N. Cruz et al., (2007)** investigated the effect of ultrahigh pressure homogenization at 200 and 300 MPa on soymilk. A soymilk base product and ultrahigh temperature treated soymilk were compared with UHPH treated soymilk. UHPH reduced initial counts, spores and enterobacteria counts. Particle size analysis evidenced the intense reduction of particle size caused by UHPH, although the formation of aggregates was detected at 300 MPa. Treated soymilk showed the lowest values of L*, a* and b* coordinates. UHPH processed samples were more stable (showed less particle settling) than BP and UHT soymilks and these differences were also observed at days 30 and 60 of storage at 4°C.
- **N.K.K. Kamizake at el., (2016)** investigated the influence of soybean aging on the physicochemical properties of soymilk and tofu. Two aging conditions were adopted: accelerated aging (84% relative humidity and 30°C, up to 9 months) and natural aging (ambient temperature and relative humidity, up to 18 months) and a control condition (47% relative humidity, 20°C). Tofu was coagulated with MgSO4. Optimum coagulant concentration decreased with increasing coagulation temperature and soybean aging time, Showed positive correlation with total solids, protein, ash, and Ca, Mg and P contents of soymilk.
- **k.anbarasu and g.vijayalakshmi (2007)** Hence an attempt was made to improve the shelf life using extracts of tulsi (Ocimums anctum) commonly available in rural areas. Tofu was

prepared traditionally using MgCl2: CaSO4 as coagulating agents. Aqueous extract of Ocimum sanctum (tulsi) was added during the preparation and storage of tofu to prolong its shelf life

5. Methodology

1. Extraction of soymilk and production of plain tofu from selected varieties of soybean seeds.

a. Soybean seeds:

Different varieties of soybean seeds will be used for the production of plain and probiotic tofu as the quality of tofu is depends on the variety of soy seeds. Soybean seeds will be procured from soybean research institute, Indore. Different varieties of soybean seeds will be selected on the basis of high protein content and high yield.

b. Extraction of soymilk from different varieties of soybean seeds:

Soymilk will be produce by the method described by (Shin, 2014) with some modifications. One hundred grams of soybeans will first rinse and soaked in 1000 mL of distilled water for 16 h at room temperature. Soaked soybeans seeds will be drain, rinse, and grind in blender for 3 min on high speed with boiling water. The water will add to make a total of 1100 g of soybean slurry. The ratios of dry soybean to water will 1:10 on weight basis. The slurry will filter through 4 layers cheesecloth to separate the 880 mL soymilk from the hulls and other insoluble materials. Seven hundred and fifty milliliters of soymilk will heat to boiling for 10 min and then boiling water will be added to maintain 750 mL of soymilk.

c. Preparation of plain tofu:

Tofu will be preparing follow pervious methods with some modifications (Chang et al., 2003: Lee & Kou, 2011). The 750 ml soymilk will be pasteurizing for 10 minutes at the temperature of 80-85°C. Probiotic tofu will prepare by coagulating the soymilk using 0.5% calcium sulphate and shell powder. The whole mixture will mildly mix and left for 10 minutes so as protein could coagulate. The soybean curd will transfer to a tofu-forming box line with 2 layers of cheesecloth and pressed and will be stored at 4°C.

2. Production of probiotic tofu by using different strains of *Lactobacillus acidophilus, L. Casei, L. paracasei.*

a. Probiotic cultures:

Probiotic strains of *Lactobacillus (LAB): Lactobacillus acidophilus, Lactobacillus casei and Lactobacillus paracasei* will be used to increase the stability and quality of tofu, which has potential health benefits. Different strains of selected species of *Lactobacillus* will be procured from (NDRI) National Dairy Research Institute, Karnal.

b. Extraction of soymilk and probiotic tofu formation:

Soymilk from different selected soybean varieties extracted in the same way mentioned above. Extracted soymilk from different soybean varieties converted into tofu by adopting the abovementioned methods. The process of probiotic tofu formation is different from plain one as it will be inoculated with different strains of *Lactobacillus*. In the process of formation of probiotic tofu, soymilk will be inoculated with different strains of LAB procured from NDRI, Karnal.

3. Physical, chemical, microbiological and sensory

a. Physical analysis

Yield:

Tofu yield was recorded and expressed as weight (g.) of tofu obtain from 100 g dry soybean seeds used for tofu preparation (Lee & Kou 2011) Tofu yield will be recorded as weight of tofu obtain from 100 g dry soybean seeds used for tofu preparation.

Texture profile:

Texture of tofu was determined through texture profile analyzer Ce Li et al (2017). Probiotic tofu samples will cut into 2.0 cm thick section 3.0 cm length and width and equilibrate to room temperature for half an hour. Three samples will compress twice to 35% deformation. The textural parameters of hardness, cohesiveness, springiness, and chewiness of each tofu sample will determine from the presented TPA curve (Zhu et al., 2016).

Microstructure:

Microstructure of tofu was determined by using Scanning Electron Microscope (Chiung-Yuan Lee et al., 2010).

b. Chemical analyses

Protein estimation

Protein content of probiotic tofu will be analyzing by the micro Kjeldahl method described by (Li et al., 2017). Take 0.55g of probiotic tofu sample in a digestion tube of instrument & add 25ml concentrate H_2SO_4 and 1-2 Catalyst stabilizers. Adjust temperature to 480°C and keep for digestion for 4-6 hours till the solution becomes blue in color. Remove the tube from 0.1N H_2SO_4 solution in a titration flask, placing of the distillation unit, attach a tube containing digest sample to distillation until the press start button to effect a metered addition of NaOH & to initiate steam distillation stops add 5 drops to yellow color. This is point now using 25ml of 0.1N HCl with 0.1N NaOH in the burette.

Once the nitrogen content will be determine it is convert to a protein content using the appropriate conversion factor: % Protein = 6.23 X% N

$$\text{Protein} = \frac{\text{blank sample} \times \text{Normality} \times 1.4007 \times 6.25}{\text{Weight of Sample}}$$

Fat content

Fat content of probiotic tofu will be determined by the Soxhlet extraction method described by (Li et al., 2017). Weigh 5 g of sample into a dry thimble. Difference in weight gives sample weight. Weight the empty soxtherm flask with boiling stone. Keep the thimble in soxtherm extractor. Pour the solvent (150 ml of hexane) into the soxtherm flask. Fix the soxtherm flask in soxtherm extraction apparatus with a reflux condenser. Keep the total arrangement of process for at least 4 hours. After 4 hours, take out the solvent from hexane and thimble from extraction apparatus. Keep the soxhlet flask in the hot air oven for 10 minutes to evaporate the solvent and cool it in a desiccator. Then weigh the flask with extracted fat.

$$\text{Fat content \%} = 100 \times (W3 - W2) / (W1 - W)$$

Where,

W - Weight in grams of empty thimble

W1 - Weight in grams of thimble with sample

W2 - Weight in grams of empty soxtherm flask

W3 - Weight in grams of soxtherm flask with extracted fat

Soluble solids content

The soluble solids content of probiotic tofu will be determine by refractometer described by (Li et al., 2017). The soluble solids content will be assayed by refractometer after filtration. Result will be recorded on a wet basis.

Calcium content

Calcium content of probiotic tofu will be determined described by (T. D. Cai 1999). 2ml of mineral solution into a 15ml centrifugal tube add 2ml of distilled water and 1ml of 4% ammonium oxalate solution and mix thoroughly and leave overnight. Again the contents are mixed and centrifuged for 5min at 1500rpm. The supernatant liquid is poured off and the centrifuge tube is drained by inverting the tube for 5min on a rack (care should be taken not to disturb the precipitate). The mouth of centrifuge tube is wiped with a piece of filter paper. The precipitate is stirred and the sides of the tubes are washed with 3ml dilute ammonia. It will be centrifuged again and drained as before. The precipitate will be washed once more with dilute ammonia to ensure complete removal of ammonium oxalate. The precipitate is dissolved in 2ml of 1N H_2SO_4. Blank: Take 2ml of 1N H2SO4 heat and titrate against KMnO4 solution till pink color is obtained.

Formulae: 1ml of 0.01N KMnO4 is equivalent to 0.2004 mf of Ca. Calcium content will be calculated.

Moisture content:

The moisture content of probiotic tofu will be analyzing by the vacuum oven method described by method (Veronica A et al., 2007). The moisture content of probiotic tofu will determine by drying 5 g of freshly prepare probiotic tofu will be place in aluminium vessel, weight and dry at 105°C in a vacuum oven. After that desiccator containing calcium chloride and phosphorous pentoxide until lost the water and is weight again. The dry sample will weight, and the moisture content will calculate using the following equation:

$$\text{Moisture \%} = \frac{(w2-w1) - (w3-w2)}{(w2 - w1)}$$

Where,

W1 = Initial weight of aluminium vessel (g)

W2 = Weight of the aluminium vessel with sample before drying (g)

W3 = Weight of the aluminium vessel with sample after drying (g)

pH measurement

The pH of probiotic tofu will determine by pH meter (Li et al., 2017).

c. Microbial Analysis

Survival of probiotic bacteria in tofu will be determined described by (Ong et al., 2006). Tofu samples (10g) will be take aseptically in jar, homogenized with 90 mL of sterile peptone water and decimal dilutions in sterile peptone water will be prepare. Obtain slurry for the dilution and subsequent serial dilutions will be performing in 0.15% (w/v) peptone and water solution. Appropriate dilutions will be pour-plates.

The probiotic microorganisms Lb. acidophilus, Lb. casei and Lb. paracasei will be enumerate with various selective media under anaerobic incubation at 37°C for 72 h. MRS-vancomycine agar use for the selective enumeration of Lb. casei and Lb. paracasei will be prepare by adding 2mL of 0.5mg mL-1 vancomycine (Sigma) solution to 1L of molten MRS agar just before pouring to obtain 1 mgL¬1 of final concentration. MRS-sorbitol agar will be used for the selective enumeration of Lb. acidophilus will be prepare by adding 10mL of membrane filtered sterile 10% solutions (w/v) of sorbitol (Sigma) to 90 mL of molten MRS agar just before pouring. The plates will incubate anaerobically at 37°C for 72h.

d. Sensory analysis

Fresh tofu was subjected to sensory evaluation based on the method proposed by (Fasoyiro et al., 2014). Probiotic tofu will cut into cubes and place on a plastic plate with a random for sensory evaluation will body and texture, flavor, taste, color and appearance, sweetness, and overall acceptability. The result will recorded using with a 9 point hedonic scale where, 1= dislike extremely, 2= dislike very much, 3= dislike moderately, 4= dislike slightly, 5= neither like nor dislike, 6= like slightly, 7= like moderately, 8= like very much and 9= like extremely for each sample.

4. **Comparative study of plain, probiotic and locally available tofu.**

A comparative study of plain, probiotic (alone and combination) and locally available tofu will be done on the basis of physic-chemical, microbiological and sensory analysis.

5. **To explore methods to extend the shelf life of probiotic tofu.**

The shelf life of probiotic tofu will be determined by method described by (Kim et al., 2007). Shelf life of any product is a critical factor. Previously some techniques and methods were used to extent the shelf life of food products. One of our aims of this research is to explore methods to extend the shelf life of probiotic tofu. Although, fermentation process increases not only the nutritional and therapeutically value but enhance the shelf life as well. To extend the shelf life of probiotic and plain tofu from various traditional methods (physical method), natural plant extracts (tulsi), (honey) and powders (Oyster shell powder, turmeric powder) will be used.

a. Oyster shell powder for extended the shelf life of probiotic tofu

Tofu was prepared by a modification of the methods proposed by Park, Nam, Jeon and Oh in 2003. Extracted soymilk (750mL) will be heated to boiling, and, then, mixed with 0.5 % calcium sulphate with 0.2% shell powder at 80°C, followed by being held for 15 min to coagulate. The soybean curd will transfer to a tofu-forming box line with 2 layers of cheesecloth and pressed and will be stored.

b. Tulsi used for extended the shelf life of probiotic tofu

Aqueous extract of tulsi was prepared according to the method described by Ganasoundari and others (1998). Leaves of tulsi will be collected, washed, dried in the shade, and ground to a powder. This powder will be used for extraction. 20 gm of leaf powder will be extracted with 150 mL of distilled water for 1 h at 75°C, cooled, and filtered. Extraction will be repeated in 4 times, pooled, and concentrated to 150 mL. This extract will be used for extended the shelf life of probiotic tofu.

Soymilk (750 mL) will be heated to boiling. Mixed coagulant (0.5% calcium sulphate) prepared in 70 mL of tulsi extract (5%) will be added to the boiling milk with slow stirring for few minutes, and held at room temperature for 15 min to coagulate. The soybean curd will transfer to a tofu-forming box line with 2 layers of cheesecloth and pressed and will be stored.

Therapeutic uses of tulsi

Tulsi have great medicinal properties. Medicinal properties of tulsi are all because of the secondary metabolite and essential oil present in the leaves. It is also recommended for the treatment of diarrhea, fever, cold cough, ophthalmic skin diseases and upper respiratory tract infections and for insect bite. It is also suggested as antimicrobial, antifungal, antibacterial, antimalarial, antiviral, anesthetic,

antiprotozoal and anthelmintic agents. It has antidiuretic, antifertility, anti-inflammatory and anti-stress activity (Cohen et al., 2014).

c. Honey used for extended the shelf life of probiotic tofu

Honey will be collected from local market in Agra. Soymilk (750 mL) will be heated to boiling. Mixed coagulant (0.5% calcium sulphate) 20 or 30 gm of honey will be added to the boiling milk slow stirring for few minutes, and held at room temperature for 15 min to coagulate. The soybean curd will transfer to a tofu-forming box line with 2 layers of cheesecloth and pressed and will be stored.

Honey is primarily made of water and carbohydrates. It also contains trace amounts of several minerals and vitamins. Also find niacin, calcium, copper, riboflavin, iron, magnesium, potassium and zinc in honey. Honey also contains a blend of flavonoids and phenolic acids. These are antioxidants that eliminate potentially destructive free radicals in the human body (K. P. Sampath Kumar et al 2010).

Therapeutic uses of honey

There are many therapeutic uses of honey such as fight acne, combat infection, fight colds, treat burns, reduce cholesterol, clear bladder infection, recede arthritis pain, sooth toothache, clear sinuses, help fertility, aide digestion, fight fatigue, help with weight loss and strengthen immune system (Kumar et al,. 2010).

d. Turmeric powder used for extended the shelf life of probiotic tofu

Turmeric powder will be collected from local market in Agra. Soymilk (750 mL) will be heated to boiling. Mixed coagulant (0.5% calcium sulphate) 5 or 10 gm of turmeric powder will be added to the boiling milk slow stirring for few minutes, and held at room temperature for 15 min to coagulate. The soybean curd will transfer to a tofu-forming box line with 2 layers of cheesecloth and pressed and will be stored.

Therapeutic uses of turmeric powder

Turmeric powder is helps for fighting infections and some cancers, reducing inflammation, and treating digestive problems. Turmeric has long been used in both Ayurvedic and Chinese medicine as an anti-inflammatory, to improve intestinal flora, eliminate worms, relieve gas, to treat digestive and liver problems, gallbladder, skin diseases, and wounds. It is being rich in iron is useful in anemia,

useful in the treatment of measles, beneficial in soreness of the eyes**.** Also used for sprains, burns, cuts, bruises, insect bites and itches, for soothing action in cough and asthma, as antibacterial and anti-fungus, and in any condition of weakness or debility (Bhowmik, Chiranjib, Kumar, 2009).

e. physical methods used for extended the shelf life of probiotic tofu

Physical method is a technique used to prevent food from spoiling for include (thermal processing and non-thermal processing) will be used for extended the shelf life of probiotic tofu.

High temperature processing

There are basic two temperatures for food preservation to kill and inhibit the growth of microorganisms and increasing the shelf life of food product, (pasteurization and sterilization) in which foods are treated at about 130°c to 135°c for 2-3 sec. or 121°c (250°F) to 132°c (270°F) hot filling, in which liquid foods and juices are boiled before being placed into containers; and steam treatment under pressure.

Low temperature processing

Low temperatures reduce the growth rates of microorganisms and slow many of the physical and chemical reactions that occur in foods. Low temperatures (-4°c) and (-18°c) are used to preserve and extend the shelf life of food by lowering microbial activity through the reduction of microbial enzyme activity.

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